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<p>Preliminary data indicated some breast cancer patients who are candidates for high dose therapy requiring prior collection of a cytokine-mobilized blood stem cell harvest for reinfusion to restore hematopoiesis, respond poorly to mobilization. This makes collection of an adequate harvest inconvenient, prolonged and costly. Poor mobilizers appear to have a circulating inhibitor of mobilization which can be assayed in a mouse model. When injected before cytokine administration mobilization is significantly decreased. The goal of this project was to provide proof of principle of this hypothesis by assaying heparanized plasma from poorly mobilizing breast cancer patients as compared to good mobilizers (patients and normal donors) and begin to attempt to identify this inhibitor. Samples from 54 patients have been collected for assay. Initial studies indicated that mobilization of blood stem cells is more complex than previously appreciated and their may be early (days 1-2) and later (days 4-5) mobilization, the former differentially forming more differentiated progenitor cells (CFC-HPP and GM) and the latter more primitive (CD34+) stem cells. The mechanisms may be different. The assay has been refined to provide a more comprehensive evaluation of these possibilities before evaluating the remaining clinical samples. Employing serum amyloid A as a surrogate of IL-1 IL 6 and TNF<math>\alpha</math> activity eliminates these as candidates for the inhibitor. Potentially inhibition of early mobilization involves a chemokine inhibitor which will be evaluated next along with the remaining clinical samples.</p>			
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***"BC 961628: Inhibition of Stem Cell Mobilization in Breast Cancer Patients by a Circulating Factor"***

**5. Introduction:**

**Statement of work revised**

Year 1: In order to demonstrate proof of principle:

- a. Confirm preliminary data that injection of human plasma from poor blood stem cell mobilizers into mice prior to mobilization with cytokines (with appropriate controls) blocks mobilization (Aim #1).
- b. Commence recruitment of breast cancer patients with Stage II, 4-9+lymph nodes, Stage II-III $\geq$ 10+lymph nodes and metastatic disease to donate a 10m ml blood sample. Plasma will be stored for subsequent assay employing normal donor plasma as the comparative control.
- c. Once the mobilization status (good, poor, intermediate) of patients recruited has been determined on the basis of the number of CFU-GM progenitor cells and CD34+ stem cells in the harvest and/or the number of leukaphereses required to achieve an adequate harvest, has been determined, patient samples will be identified for evaluation, compared to normal donor plasmas, in the mouse assay as in Work Statement #1 above (Aim #1).

**6 Body**

**a. Acquisition of Clinical Samples**

Up to the current time, 54 clinical samples have been acquired for assay of inhibitor(s) of blood stem cell mobilization. These have been obtained in a double blind fashion in that co-investigator Kessinger maintains the data base on the categorization of these samples and this information is not known at the time of performing the inhibitor assay. Dr. Kessinger does not know the results of the inhibitor assay until she has categorized the donor as a good or poor mobilizer.

In addition to acquiring samples from breast cancer patients, samples have been acquired from normal donors who have not been exposed to radiation therapy or chemotherapy in order to attempt dissect the individual roles, if any, of tumor versus prior therapy. In order to provide a more comprehensive analysis of the

patient samples most have been held for analysis until studies of refinement of the inhibitor assay have been completed (see below).

***b. Refinement of the Inhibitor Assay***

The assay as developed originally involved injection of potential inhibitor into mice 10 minutes prior to cytokine administration. Mobilization was assayed the next day by enumerating colony forming cell numbers (GM-CFC) and (HPP-CFC) in the spleen. There are two potential disadvantages to this assay as originally formulated. The first is the practical aspect in that it takes 10 days to acquire the colony data. This is inconvenient if one wished in the future to intervene clinically by, for example, changing the mobilizing cytokine used for a patient, who appears to be a poor mobilizer. If flow cytometric techniques could be employed to enumerate mobilized stem cells, this could reduce the assay time to a few hours. A second potential problem concerns the interpretation of the assay of suppressed colony forming cell numbers. While the simplest interpretation of this observation is that mobilization of colony forming cells was inhibited, formally, the possibility that the inhibitor suppression differentiation of colony forming cells from more primitive stem or progenitor cells cannot be excluded. This is a property of some chemokines (see Rollins, p919, 1997). Employing a flow cytometric assay of (primitive) stem cells as well as assaying colony forming cells would address this issue. Consequently, the development of a faster, more efficient flow cytometric assay of stem cells was pursued. Development of this assay has proved to be challenging because of a number of unanticipated and previously undescribed aspects of the mobilization of blood stem cells in mice. In man, stem cells express the CD34 marker. Much less is known about the expression of this marker in mice and it does not appear to track well with colony forming cell assays. In mice, historically, the SCA-1 marker has been, used in mice (of specific strains and specified age) to mark primitive stem cells capable of hematopoietic reconstitution of lethally irradiated mice when transplanted in low numbers eg 1, 10 or 30 cells. However, this only applies to bone marrow stem cells. This marker cannot be applied in blood or spleen stem cell assays i.e. to assess mobilized stem cells, because other cells in these tissues express sca 1. Conveniently, the use of CD34 with a CD45 back gating strategy as in the ISHAGE protocol for man appears useful as does the combination of assay of CD34 with c-kit.

There are some additional puzzling observations from these attempts to improve the assay of mobilization of blood stem cells. The assay of inhibitor was originally performed after about 18-24 hours. In studies of mobilizing agents, some, especially chemokines, such as IL 8 (Rollins 1997) which act through small G proteins (Hall, 1998) as well as cause rapid mobilization within a few hours. This may be the target of mobilization which is inhibited. It is not clear that this

mechanism of mobilization is the same as mobilization observed after 5 days which is when stem cells are usually collected clinically. The implication of this observation is that the inhibitor of mobilization may be a chemokine inhibitor. The other important factor which influences the assay in mice is that the pattern and extent of mobilization in mice is strain dependent and genetically determined in a polygenic manner. The assay originally employed Balb/c mice which Metcalf and colleagues (Roberts et al. 1997) have shown are good, albeit slow, mobilizers. C57 B1 mice mobilize poorly. In contrast DBA mice mobilize well and more rapidly than Balb/c mice. If the inhibitor acts on more rapidly mobilized stem cells then DBA mice might represent the best assay system. This question is being investigated. The initial results hint that the reason DBA mice are rapid mobilizers is that early (day 1-2) mobilization is prominent. Later (day 4-5) mobilization also occurs. In contrast, Balb/c mice appear less able to mobilize early but mobilize well at the later times (days 4-5). It appears that it will be necessary to determine the effects of inhibitor on both of these phases of mobilization since these may have differing mechanisms and may respond to different inhibitors. As noted above, inhibitors of adhesion molecules can mobilize and adhesion molecule expression (Vermeulen et al. 1998; Yamaguchi et al. 1998) as well as stem cell pheno type (Habibian et al. 1998) change with the cell cycle status of the stem cells. Because of these inter-relationships it was felt to be essential to increase the sophistication of the inhibitor assay so that it covers both primitive and more differentiated stem cells, and early and late mobilization.

*c. Preliminary Results of Inhibitor Assay*

A small number of samples were analyzed in the inhibitor assay before the assay was refined. Figures 9a and 9b show the same sample assayed after about 24 hours in Balb/c and DBA 2 mice using the flow cytometric assay. Inhibition was evident in the DBA 2 mice but not in the Balb/c mice and this might be because the early phase of mobilization may not be as evident in the Balb/c mice 2 (see above). When assayed as colony forming cells inhibition in Balb/c mice was evident as observed previously (Kessinger and Sharp 1998). Further analysis of inhibitor was then deferred until the assay was refined (see above).

*d. Identification of the Inhibitor(s)*

External reviewers of proposals and manuscripts describing this inhibitor expressed their mutually exclusive confidence that the inhibitor, without doubt was TNF $\alpha$  in one case and TGF $\beta$  in the other. TGF $\beta$  exists in multiple isoforms and latent and active forms and is more difficult to assay. Consequently, it was considered initially to be the best strategy to test the hypothesis that it was TNF $\alpha$ . An indirect method to accomplish this and test if IL1 or IL6 are also involved, is to

assay the presence of serum amyloid A (SAA). This acute phase protein is induced by these cytokines. Since these cytokines can be elevated in patients due to their tumor and/or prior therapy it was decided a more controlled situation was to compare plasma from part body irradiated versus sham-irradiated mice with the assistance of Dr. Tom MacDonald and Annika Webber. SAA levels were similar in irradiated versus sham-irradiated mice. This is in agreement with data from Greenberger's group which indicates that the SAA response is not induced until about one week post irradiation. (Goltry et al. 1998) This suggests that it is unlikely that the inhibitor of blood stem cell mobilization is IL-1, IL-6 or TNF $\alpha$ . Two dimensional electrophoretic assays of plasma proteins as well as ELISA assays are currently underway in an attempt to confirm these observations. Currently, as noted above, a likely potential candidate inhibitor is a chemokine inhibitor which inhibits a chemokine involved in mobilization. Since over 70 chemokines have been identified (updated from Rollins 1997) it will likely take some time to identify which might be involved, unless serendipity intervenes.

e. Figures and Figure Legends

SAA in Mouse Sera		
		SAA ug/ml
DBA Plasma	Normal	11.2
	Irradiated	10.8
Balb/c Plasma	Normal	9.1
	Irradiated	15.0

**Figures 1a-d** illustrate cytokine mobilization grossly in DBA 2 mice in terms of increased blood cell counts, spleen weight and cellularity. There is little impact on femoral bone marrow cellularity which decreases slightly until time.

**Figures 2-4** illustrate what appears to be two phases of mobilization in DBA2 mice. When studied using an ISHAGE-like flow cytometric protocol for evaluating human stem cells adapted to mice (enumeration of CD45+CD34+cells) elevations of positive cells are seen in bone marrow on day 3 and in blood and spleen on day 4. This appears to precede significant elevations of HPP and GM-CFC per spleen on day 5. This is the typical mobilization pattern we have observed previously in Balb/c mice (Sharp et al. 1998).

However, in DBA2 mice there is an additional mobilization component evident in blood and spleen HPP and GM-CFC at day 2. This early mobilization is most evident as mobilized progenitor colony forming cells but not evident for more primitive CD45+CD34+cells, although the possibility that this was missed because it occurred on day 1 cannot be excluded.

These results suggest there are two components to mobilization, an early

component most evident at day 2 and a later component evident at days 4-5 in DBA2 mice. The early component is most evident in terms of colony forming cells. Potentially, these two components have different underlying mechanisms of mobilization.

**Figures 5a-d** illustrate mobilization grossly in Balb/c mice. Blood cellularity on days 1 and 2 was significantly elevated. Mobilization was evident by increasing spleen weight and cellularity up to day 5. Femoral bone marrow cellularity decreased on day 1 but largely was unaltered during mobilization (Fig 5d).

**Figure 6a** shows mobilization on day 1 and days 3-5 in Balb/c mice measured flow cytometrically. The observation of mobilization of CD 34 positive cells into blood on day 1 in this strain is one reason for suspecting this may have been missed in DBA2 mice which were not studied on day 1 (see above). Mobilization was also seen in the spleen at day 4. Generally femoral bone marrow stem cell numbers appeared slightly depressed during mobilization. Figures 7 and 8 show mobilization in Balb/c mice in terms of HPP- CFC in blood and spleen and GM-CFC blood and spleen. The effects are most evident at days 2-3 in blood and days 3 and 5 in spleen. The increased colony number seen at day 2 in DBA2 mice was much less evident. Colony numbers in femoral bone marrow showed significant variability but no discernable pattern and fell generally in the normal control range.

**Figure 9a** shows inhibition detected in DBA2 mice using the flow cytometric assay after approximately 24 hours. This same sample did not appear to inhibit in Balb/c mice. However, because of the lower early mobilization of Balb/c mice this may not be the best strain in which to evaluate early mobilization.

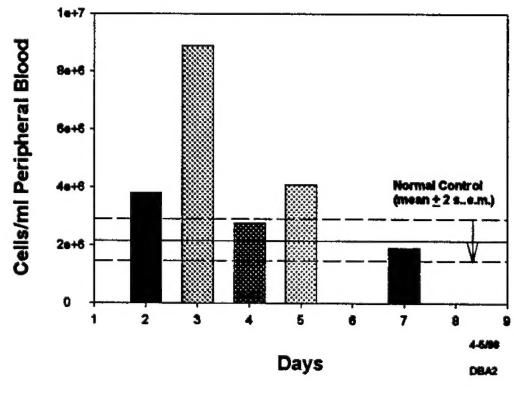
**Figure 10** shows that this sample inhibited in Balb/c mice when assayed using the colony forming cell assay as reported previously. Potentially this indicates that colony forming cells show a more pronounced early mobilization than more primitive (flow cytometrically detected) stem cells.

#### *f. Future Studies*

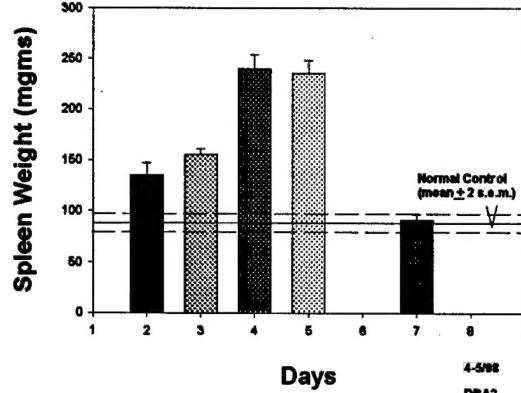
The plan is to assay the clinical samples employing the newly developed flow cytometric as well as colony assays applied to assess both early (day 2) mobilization and later (day 5) mobilization in both DBA 2 and Balb/c mice. Experiments will continue to identify the inhibitor, focussing currently, on chemokine inhibitors.

**Figure 1.**

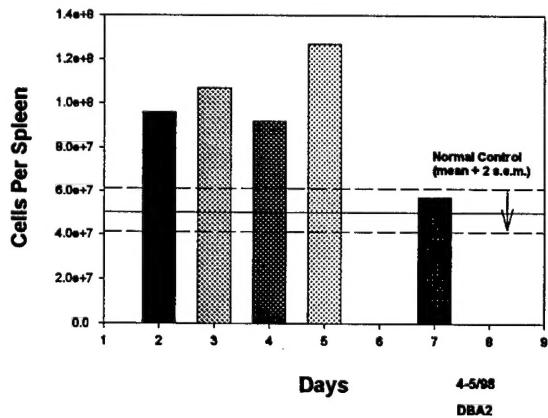
**A. Cellularity After Mobilization With  
G-CSF (15 ug/kg) + EPO (500 U/kg)**



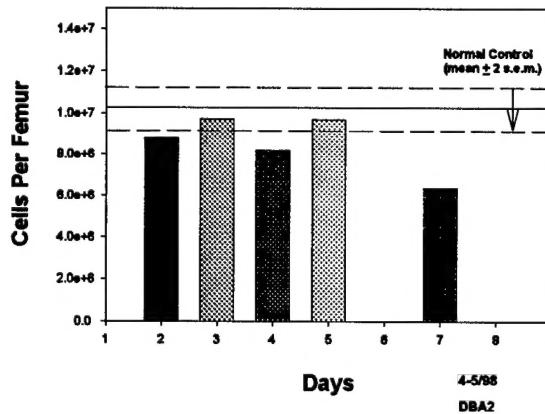
**B. Spleen Weight After Mobilization  
With G-CSF (15 ug/kg) + EPO (500 U/kg)**



**C. Cellularity After Mobilization With  
G-CSF (15 ug/kg) + EPO (500 U/kg)**

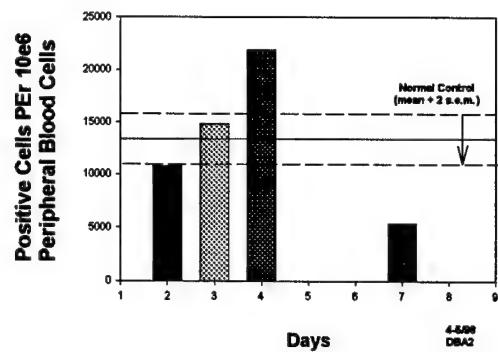


**D. Cellularity After Mobilization With  
G-CSF (15 ug/kg) + EPO (500 U/kg)**

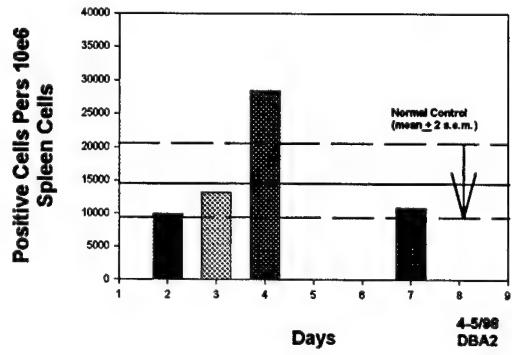


**Figure 2.**

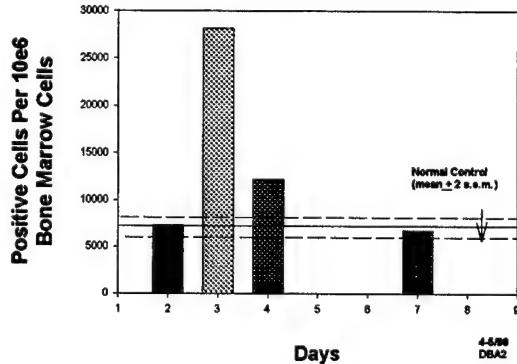
**A.**  
**CD45+/34+ Subpopulations Following  
Mobilization With G-CSF (15 ug/kg) + EPO (500 U/kg)**



**B.**

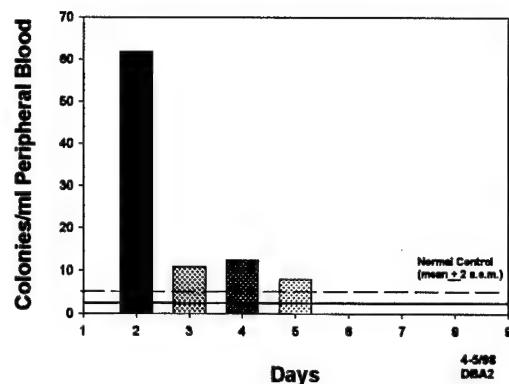


**C.**

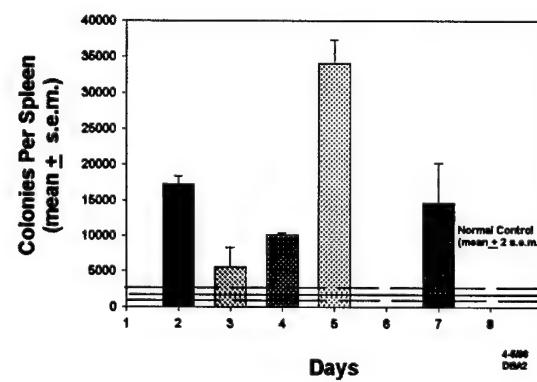


**Figure 3.**

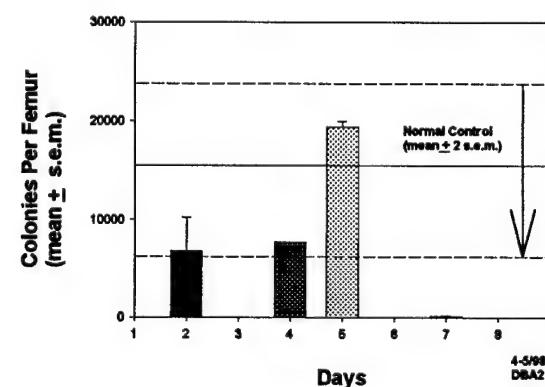
**A.**  
**HPP Colonies After Mobilization  
With G-CSF (15 ug/kg) + EPO (500 U/kg)**



**B.**

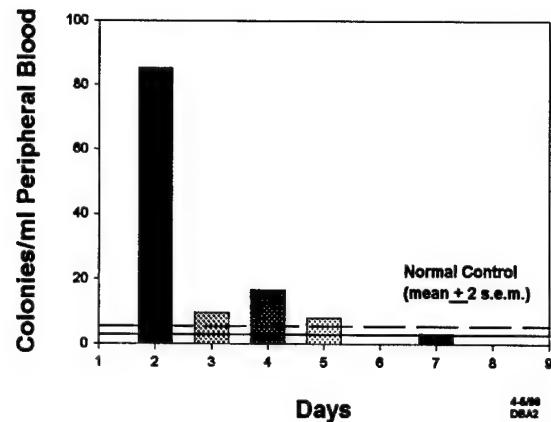


**C.**

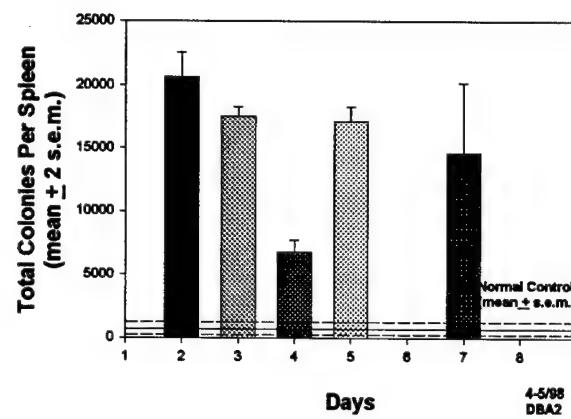


**Figure 4.**

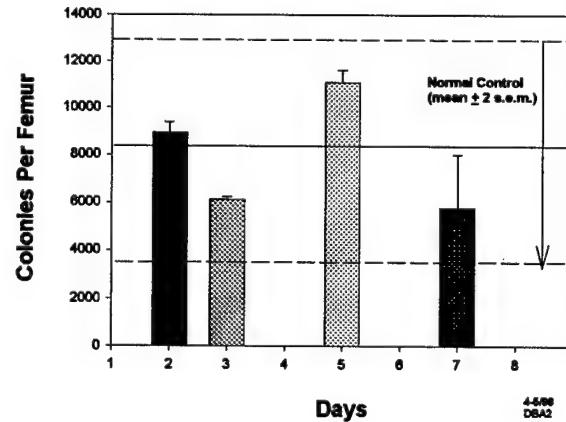
**A. CFU-GM Colonies After Mobilization  
With G-CSF (15 ug/kg) + EPO (500 U/kg)**



**B.**

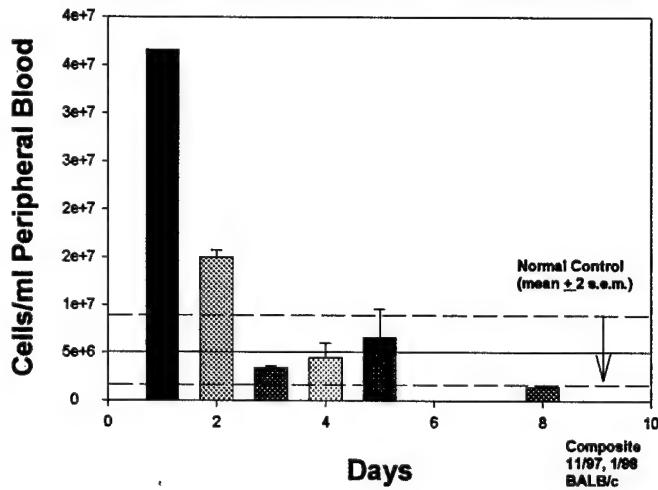


**C.**

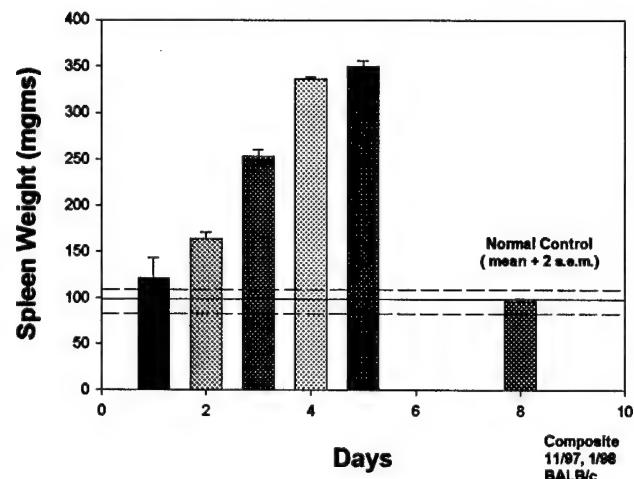


**Figure 5.**

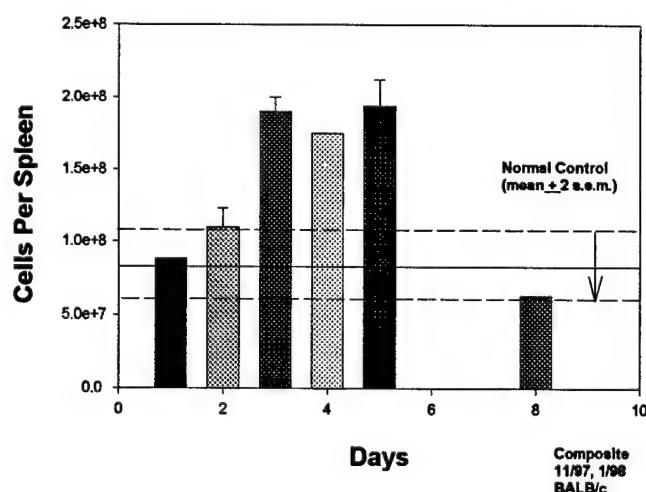
**A. Cellularity After Mobilization With G-CSF (15 ug/kg) + EPO (500 U/kg)**



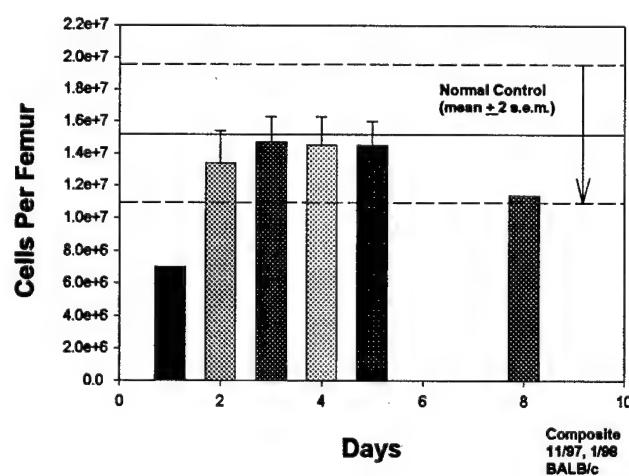
**B. Spleen Weight After Mobilization With G-CSF (15 ug/kg) + EPO (500 U/kg)**



**C. Cellularity After Mobilization With G-CSF (15 ug/kg) + EPO (500 U/kg)**



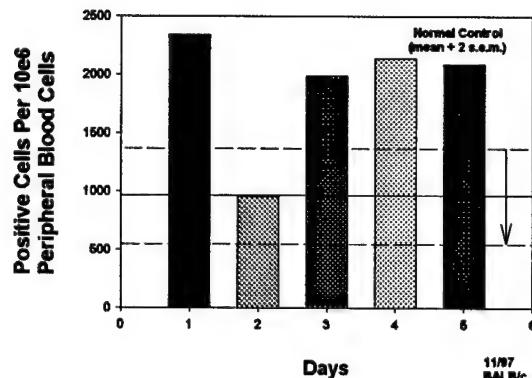
**D. Cellularity After Mobilization With G-CSF (15 ug/kg) + EPO (500 U/kg)**



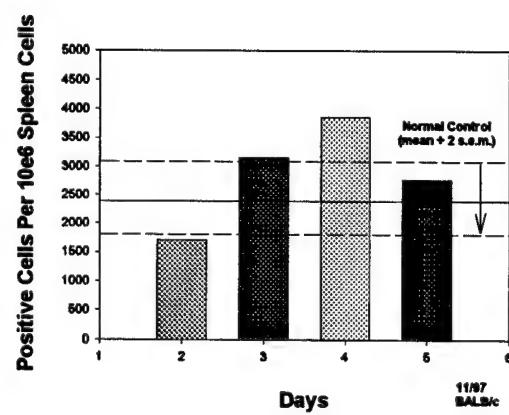
**Figure 6.**

**CD34+ Subpopulations After Mobilization  
With G-CSF (15 ug/kg) + EPO (500 U/kg)**

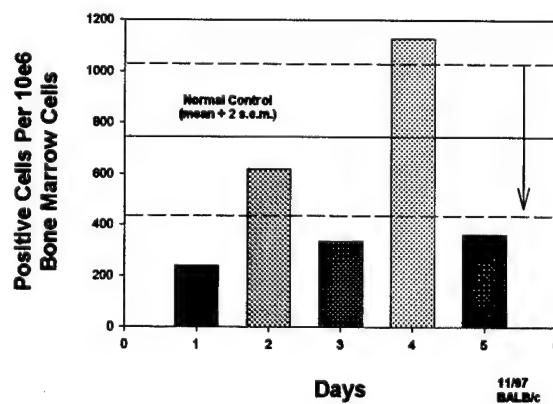
**A.**



**B.**

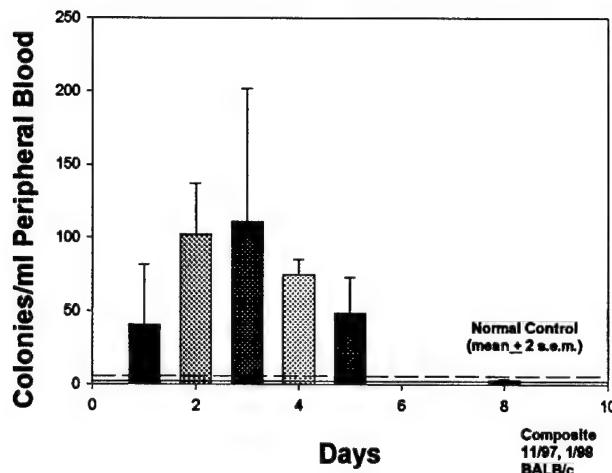


**C.**

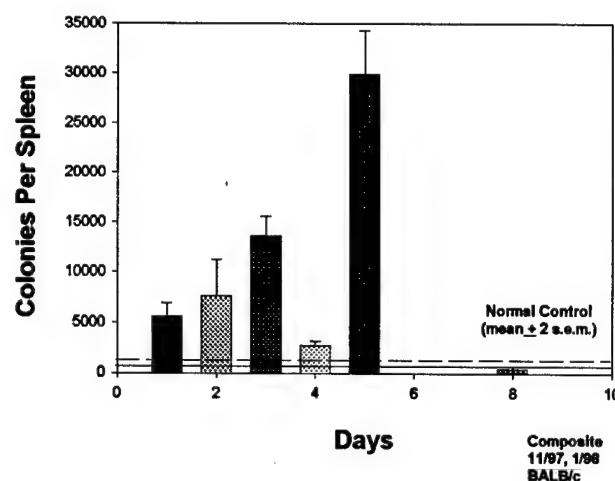


**Figure 7.**  
**HPP Colonies After Mobilization**  
**With G-CSF (15 ug/kg) + EPO (500 U/kg)**

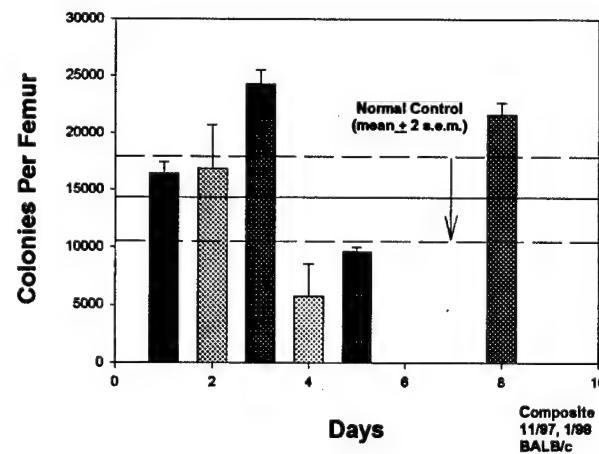
**A.**



**B.**



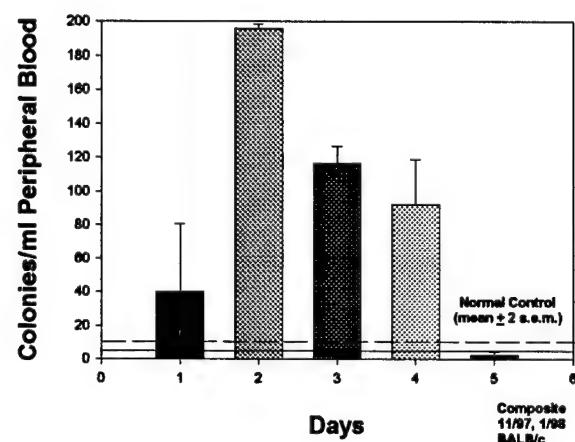
**C.**



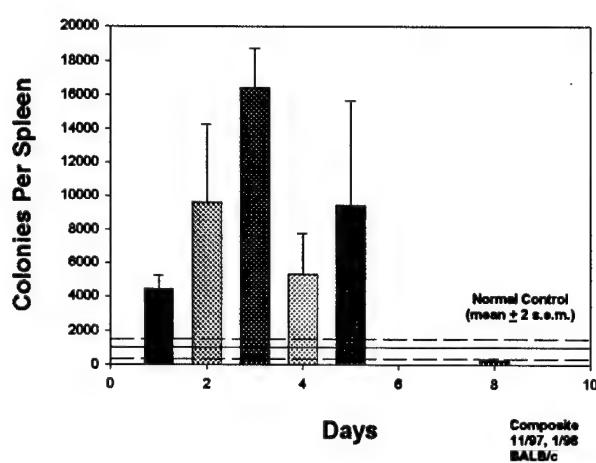
**Figure 8.**

**GM-CFC Colonies After Mobilization  
With G-CSF (15 ug/kg) + EPO (500 U/kg)**

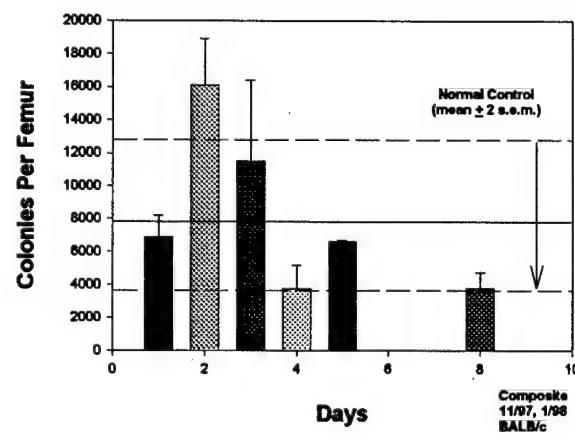
**A.**



**B.**



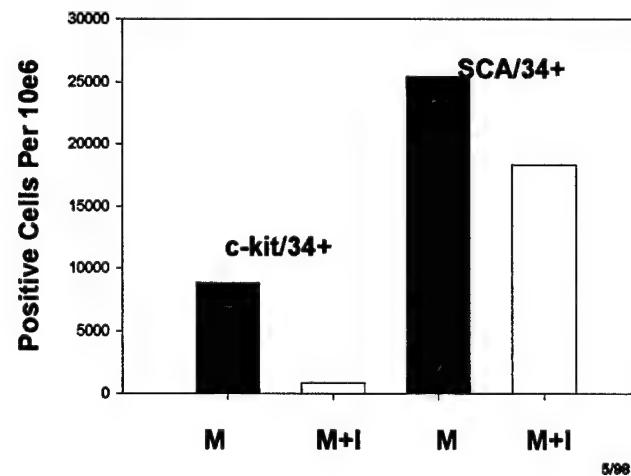
**C.**



**Figure 9.**

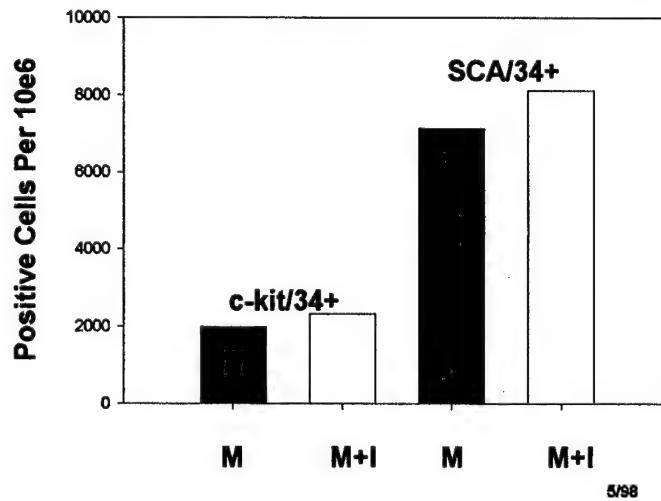
**A.**

**Flow Cytometric Subpopulations  
in Peripheral Blood of DBA2 Mice**

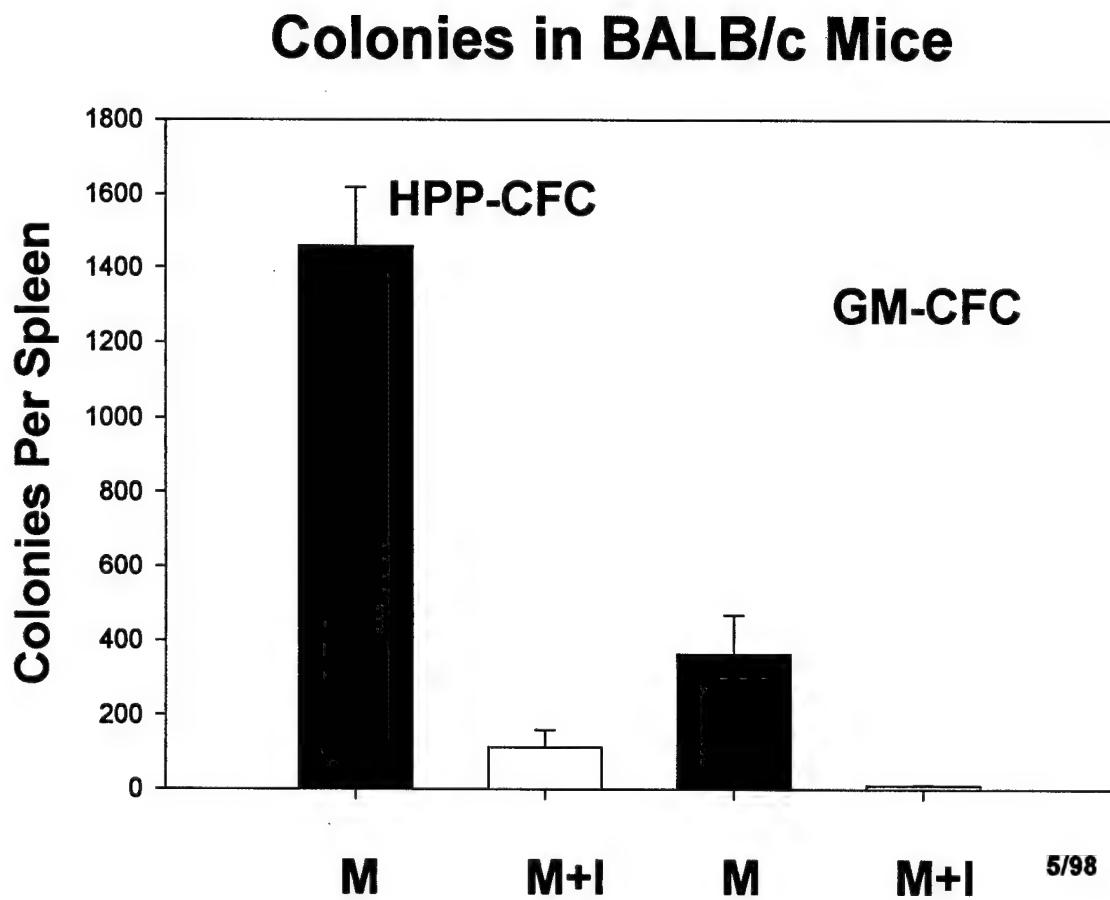


**B.**

**Flow Cytometric Subpopulations  
in Peripheral Blood of BALB/c Mice**



**Figure 10.**



## **7. Conclusions**

Currently, limited initial data indicate that poorly mobilizing breast cancer patients have a circulating inhibitor of mobilization. However, blood stem cell mobilization appears to be a complex process which may have two (an early and late) components. The type of stem mobilized early versus late may be different, with more differentiated progenitors mobilized early and more primitive stem cells later. Whether the circulating inhibitor in breast cancer patients inhibits one or both of these components remains to be determined in the next year of these studies.

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Appendix 9a

## Concurrent Partial Body Radiation Prevents Cytokine Mobilization of Blood Progenitor Cells: An Effect Mediated by a Circulating Factor

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### ABSTRACT

Mobilization of stem and progenitor cells into blood, which facilitates the collection of blood-derived autograft and allograft products, can be accomplished with administration of myelosuppressive chemotherapy, hematopoietic growth factors, or both. Autologous donor indifference to mobilization attempts has been correlated with prior administration of chemotherapy and radiation therapy. To investigate whether concurrent administration of radiation therapy inhibits mobilization, five daily injections of a potent combination of mobilizing cytokines, 500 U/kg erythropoietin (EPO) plus 15  $\mu$ g/kg G-CSF, were administered each morning to Balb/c mice. Each afternoon, a 2 Gy fraction of Co-60 radiation was administered to either the lower limb or the upper or lower hemibody. Each day, mice were necropsied, and blood stem cell mobilization was determined by assaying the number of hematopoietic colony-forming cells in the blood and in the spleen. Unirradiated cytokine-injected mice showed a significant mobilization effect evident as increased colony-forming cells in blood and spleen compared with saline-injected unirradiated controls. The irradiated mice showed markedly inhibited or absent mobilization regardless of the part of the body irradiated. To investigate the mechanism of radiation-induced mobilization inhibition, heparinized plasma was obtained from mice whose lower bodies were irradiated with 2 Gy 18 h previously, and 0.5 ml was injected i.v. into intact mice 10 min before they received 15  $\mu$ g/kg G-CSF and 500 U/kg EPO. Unlike mice that received G-CSF + EPO only and showed mobilization of progenitors from marrow to spleen, recipients of plasma from irradiated mice before and after cytokine administration showed significantly reduced mobilization of progenitors. Thus, radiation-induced inhibition of stem cell mobilization is mediated by an unidentified circulating factor.

### INTRODUCTION

ADMINISTRATION OF HEMATOPOIETIC CYTOKINES is used increasingly clinically to produce mobilization of stem cells in normal and autologous donors who undergo blood stem/progenitor cell collections (1). Cytokine-induced mobilization occurs with varying degrees of vigor in patients and, occasionally, does not occur at all, even in normal subjects (2,3). Although risk factors, such as

prior cytotoxic therapy and the presence of metastatic disease in the marrow, incompletely predict for indifference to mobilization attempts (2), the mechanism of this indifference has not been considered or explored. This study was designed to determine if radiation administration would affect the ability to mobilize stem cells into blood. When mobilization impairment was found, further studies to identify the mechanism(s) involved were formulated.

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## MATERIALS AND METHODS

## Mice

We used young adult female Balb/c mice purchased from Charles River (Wilmington, MA). On each day of analysis, 3 mice per group were studied, the experiment was repeated three times, and the data were pooled (9 mice per group total). The mice were maintained on a 12-h light, 6:00 AM–6:00 PM, 12-h dark cycle and were provided both sterilized food and acidified (pH 2) sterile water ad libitum. They were maintained in filter-top cages in laminar airflow cabinets.

## Cytokines

Based on prior observations (4) that a combination of the cytokines erythropoietin (EPO) 500 U/kg (Ortho Biotech, Raritan, NJ) and G-CSF 15  $\mu$ g/kg (Amgen, Thousand Oaks, CA) produced much better mobilization than G-CSF alone (data not shown), this combination was selected as the mobilizing regimen.

## Radiation therapy

Preliminary experiments were performed to select radiation therapy regimens that differentially irradiated

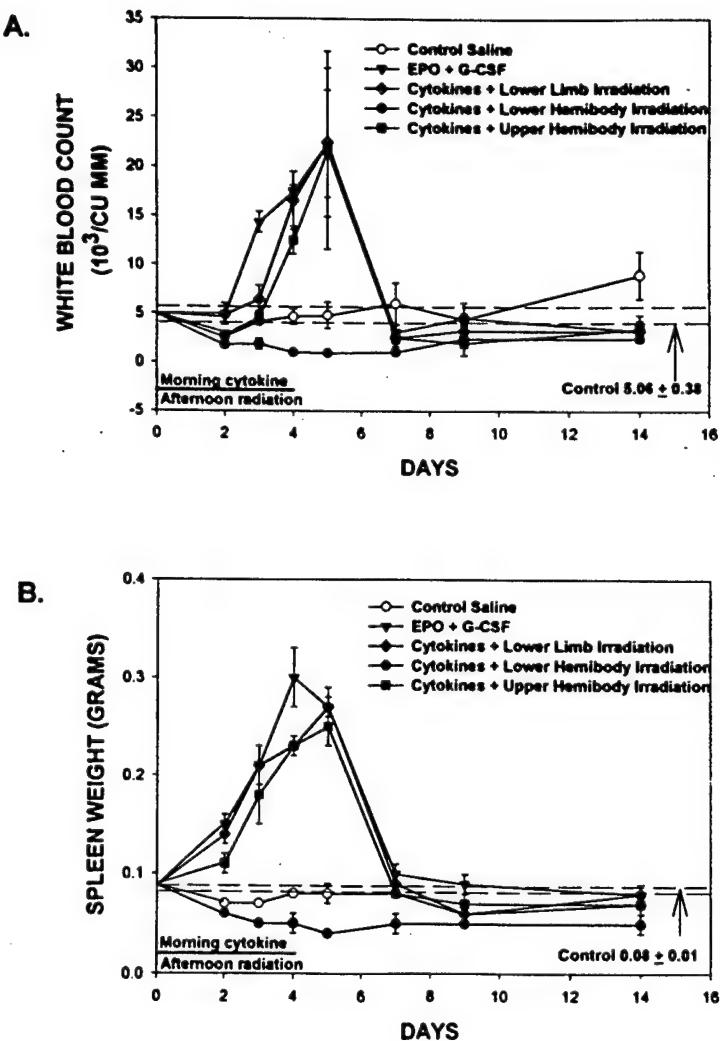
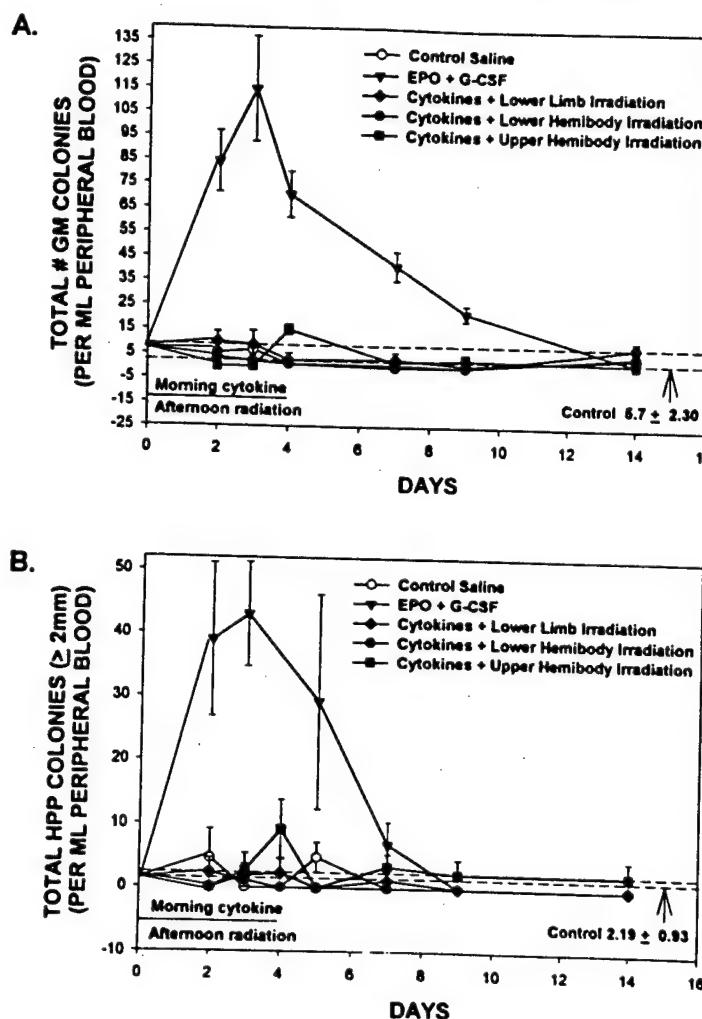


FIG. 1. Impact of cytokine administration (EPO 500 U/kg plus G-CSF 15  $\mu$ g/kg) with or without concurrent lower limb, lower hemibody, or upper hemibody radiation therapy (daily 2-Gy fractions for 5 days) on the (A) nucleated WBC count and (B) spleen weight of mice. Compared with the saline-injected control mice, WBC count and spleen weight are similarly increased in all groups except the lower hemibody irradiated mice, in which the spleen was in the irradiation field. The error bars display mean  $\pm$  standard error values for the experimental groups. The lines represent the mean  $\pm$  standard error values for the control mice.

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**FIG. 2.** Impact of concurrent radiation therapy (daily 2-Gy fractions for 5 days) to the lower limbs, lower hemibody, and upper hemibody on mobilization of stem cells into the circulation by cytokine injections (EPO, 500 U/kg plus G-CSF 15  $\mu$ g/kg), assayed as (A) GM colonies per milliliter and (B) HPP colonies ( $\geq 2$  mm) per milliliter of blood. The lack of any effect of control saline and mobilization in response to cytokine injections alone is also shown. The error bars display mean  $\pm$  standard error values for the experimental groups. The lines represent the mean  $\pm$  standard error values for the control mice.

components of the hematopoietically active tissues of the mouse. Irradiation of the femurs primarily (i.e., lower limb) involved a significant marrow volume, little of the blood pool, and not the spleen. Lower hemibody irradiation (spleen and below) irradiated a substantial marrow volume and blood pool and the spleen. Upper hemibody irradiation, including the heart and lungs but not the spleen, irradiated a very significant blood pool and a moderate marrow volume. These were selected as the irradiation regimens. The radiation was delivered as 2-Gy fractions using a Picker V90 cobalt-60 source at a dose rate of 0.67 Gy/min. All mice survived the courses of hemibody radiation therapy.

### *Cellularity and hematopoietic assessment*

The nucleated cellularity of the blood, femoral marrow, and spleen was determined, as was spleen weight, RBC count, and platelet count. The hematocrit and hemoglobin were determined using a Serono-Baker Diagnostics (Allentown, PA) System 9000 DIF Model Automated Cell Counter.

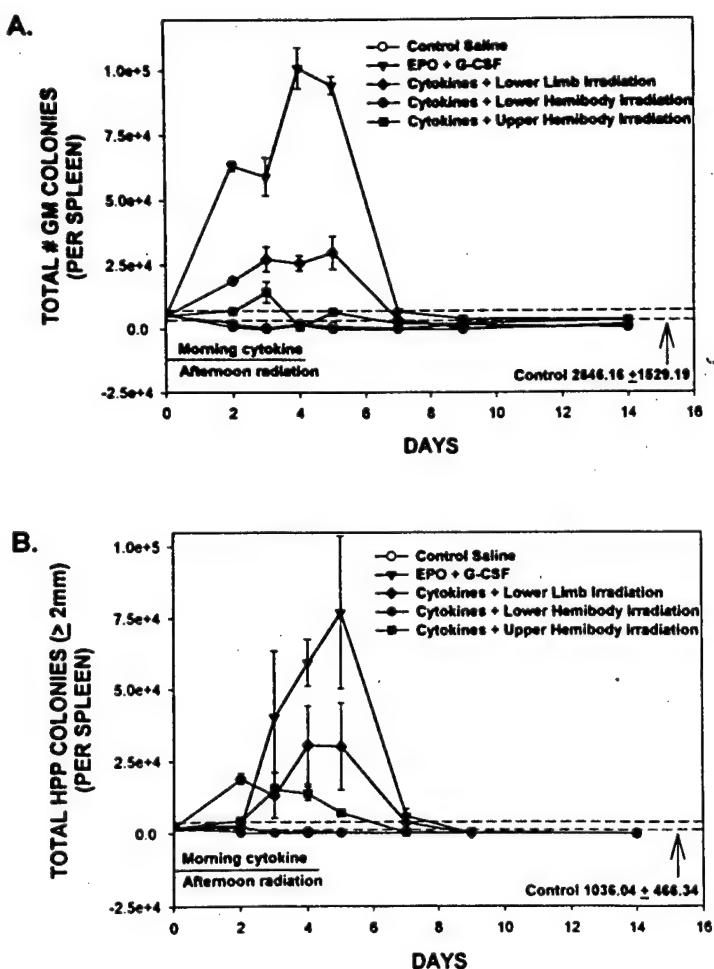
### *Hematopoietic colony assays*

Mobilization of stem cells was assessed by in vitro hematopoietic colony-forming assays: colony-forming

cells granulocyte-monocyte/macrophage series (CFU-GM) and more primitive high proliferative potential colony-forming cells (HPP-CFC). Briefly, at necropsy, blood was collected, heparinized, and separated on a gradient, and the cell content was enumerated. These cells were used for subsequent studies. To ensure an adequate number of cells for assay, especially from the blood of control mice, cells were pooled from groups of 3 mice and plated in triplicate for colony assays. Each of the means of triplicate cultures was employed to calculate the mean value for the group. Femurs were removed aseptically, and the bone marrow was flushed into HBSS without calcium and magnesium using a 1-ml syringe and a 22-gauge needle. Clumps were dispersed into a single cell suspension by repeated gentle aspiration with the needle and syringe, and the cells were enumerated and used for further studies.

Spleens were removed aseptically, and cells were gently teased from the spleens with 25-gauge needles into HBSS without calcium and magnesium. Clumps were dispersed by repeated aspiration with a 1-ml syringe without a needle. Cells were washed once and resuspended in Tris-buffered ammonium chloride (ACT) for 5 min to lyse mature RBC. After 5 min, an equal volume of complete medium containing serum was added to halt the action of the ACT. The cells were washed once and resuspended in HBSS without calcium and magnesium. These cells were then enumerated and employed in the colony assays detailed.

**HPP Assay.** Bone marrow ( $5 \times 10^4$ ), spleen ( $5 \times 10^5$ ), and blood cells ( $1.5 \times 10^5$ ) were plated in 60-mm dishes containing IMDM, 0.3% agar, 15% (FBS), 100 U penicillin, 100  $\mu$ g streptomycin, 50  $\mu$ M 2-mercaptoethanol, and 10 ng IL-3 with 10% L cell-conditioned medium or recombi-



**FIG. 3.** Impact of concurrent radiation therapy (daily 2-Gy fractions for 5 days) to the lower limbs, lower hemibody, and upper hemibody on mobilization of stem cells into the spleen by cytokine injections (EPO 500 U/kg plus G-CSF 15  $\mu$ g/kg) assayed as (A) GM colonies and (B) HPP colonies ( $\geq 2$  mm) per spleen. The lack of any effect of control saline and increases in response to cytokine injections alone is also shown. The error bars display mean  $\pm$  standard error values for the experimental groups. The lines represent the mean  $\pm$  standard error values for the control mice.

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nant M-CSF as sources of colony-stimulating activity. Dishes were incubated for 11 days at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> in air. The colonies were enumerated using an inverted microscope. Macroscopic colonies greater than 2 mm were counted as HPP colonies.

**CFU-GM.** Bone marrow ( $1 \times 10^5$ ), spleen ( $1 \times 10^6$ ), and peripheral blood cells ( $3 \times 10^5$ ) were plated in 35-mm dishes containing IMDM, 15% FBS, 50  $\mu$ M 2-mercaptoethanol, and 10 ng IL-3 with 10% L cell-conditioned medium (or recombinant M-CSF) as sources of colony-stimulating activity. Dishes were incubated for 7 days at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> in air. Groups of  $\geq 50$  cells without red cells present were counted as GM colonies.

### Data analysis

Means and standard deviations were calculated when possible. Some data, because of technical necessity, were collected as pooled specimens so that it was only possible to determine an error between assays. Nucleated cells per tissue or milliliter of blood, as well as colony-forming cells in blood, spleen, and femoral bone marrow, were enumerated. Based on determination of cellularity of the femoral bone marrow, spleen, and a unit volume (ml) of blood, these results were expressed as colonies per  $10^5$  or  $10^6$  cells. Colony-forming cells per tissue were calculated by multiplying the number of colonies per  $1 \times 10^5$  nucleated cells by the total nucleated cells in that tissue, permitting calculation of CFU-GM and HPP per milliliter of blood or per spleen or per femur.

### Experimental design

In the initial experiment, mice received daily morning injections of either saline (control) or cytokines and then

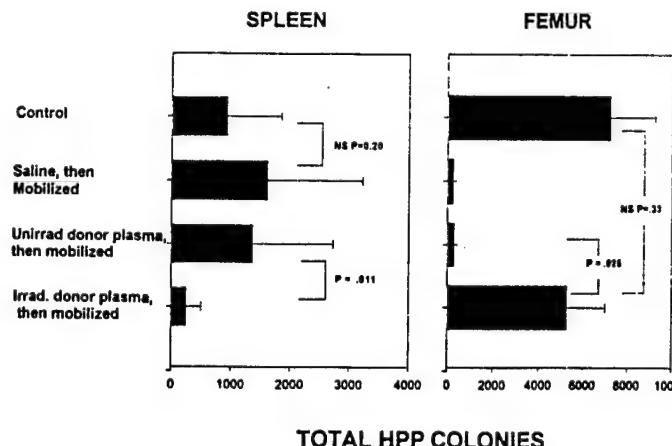
received daily (afternoon) fractions of radiation therapy to their lower limb or lower or upper hemibody for a maximum of 5 days. Groups of mice were necropsied daily and assayed for stem cell mobilization.

In a subsequent experiment, mice received an injection of mobilizing cytokines in the morning and in the afternoon received a 2-Gy lower hemibody fraction of radiation therapy. They were necropsied the following morning, and heparinized plasma was obtained. One-half milliliter of this heparinized plasma was injected i.v. into a second group of mice 10 min before they received an injection of the mobilizing cytokines. These mice were necropsied the following morning (18 h postmobilization) and mobilization assayed as described previously.

## RESULTS

Figure 1A shows the impact of partial body irradiation on the WBC count, and Figure 1B shows the effect on spleen weight. Effects on spleen cellularity were similar (data not shown). Increases in WBC count and spleen weight were evident in the cytokine-mobilized groups with and without partial body irradiation, with the exception of the lower hemibody irradiation group, in which the spleen was in the irradiation field. No other major changes in other hematologic values (platelets, hematocrit, hemoglobin, and femoral bone marrow cellularity) were noted, with the exception of a reduced cellularity of the femoral bone marrow in those mice in which this tissue was included in the irradiated field (data not shown).

Figure 2 shows the impact of partial body irradiation of mice on cytokine mobilization of blood stem cells assayed as GM colonies per milliliter of blood (Fig. 2A) and HPP ( $\geq 2$  mm diameter) per milliliter of blood (Fig. 2B). The saline control values for individual days as well



**FIG. 4.** Demonstration that injection of heparinized plasma from lower hemibody irradiated mice injected before cytokine administration almost completely blocks mobilization of stem cells from the femoral bone marrow to the spleen, which is evident when saline is injected before mobilizing cytokines (EPO, 500 U/kg plus 15  $\mu$ g/kg G-CSF). The error bars display mean  $\pm$  standard error values for the experimental groups.

as the 95% confidence limits for the colony numbers in saline-injected mice are shown. Mice injected daily with EPO + G-CSF showed a very significant mobilization of hematopoietic stem/progenitor cells into blood, with about a 20-fold increase in both GM and HPP colonies, maximal on day 3. Following cessation of cytokine injection, these values fell back to control levels over the next 3–6 days. In mice receiving a 2-Gy fraction of irradiation to the lower limbs, lower hemibody including the spleen, or upper hemibody excluding the spleen, no significant increases in stem/progenitor cells in blood were assayed at any time, indicating a very significant inhibition of mobilization.

A similar inhibition of colony formation was observed for cells obtained from the spleens of these mice (Fig. 3). Whereas following cytokine injection without radiation, GM colony number increased 35-fold and HPP 75-fold, after lower limb irradiation, the increases were less than 10-fold and 25-fold, respectively, and after upper hemibody irradiation, they were less than 2-fold and 10-fold, respectively. The effects on spleen *in vitro* colony-forming cells is relevant only for the lower limb and upper hemibody irradiated mice, as in the lower hemibody irradiated mice, the spleen was included in the radiation field, and colony numbers would be expected to be suppressed. Consequently, these results demonstrate that concurrent partial-body radiation inhibited cytokine-stimulated blood stem cell mobilization.

Figure 4 shows the results of experiments in which mice were injected either with saline (control) or cytokines (EPO + G-CSF), but in addition, 10 min before either saline or cytokine injection, they were injected i.v. with heparinized plasma (0.5 ml) obtained either from unirradiated mice or from mice that had undergone lower hemibody irradiation. The irradiation consisted of a single 2-Gy fraction, and the mice were necropsied and plasma was obtained 18 h later. The end point of this experiment was assay of GM or total HPP colonies in the spleen or femur 18 h after injection of the mobilizing cytokine. The HPP data are shown.

The GM results were similar. As expected, injection of saline or unirradiated mouse plasma before cytokine did not influence mobilization, which was evident as a reduction in the number of HPP in the femoral bone marrow and an increase in the spleen compared with non-mobilized mice. In contrast, injection of plasma from irradiated mice 10 min before injection of cytokines almost completely, and significantly, inhibited mobilization of stem/progenitor cells to the spleen, and they were maintained at untreated control levels in the femoral bone marrow. Therefore, it appears that the inhibition of blood stem cell mobilization observed in irradiated mice was mediated by a circulating factor. The characteristics of this factor are currently unknown.

## DISCUSSION

In the past, at a time when blood stem cells were collected without mobilization, radiation therapy during the days blood stem cells were harvested resulted in a poor quality of the harvest, necessitating multiple apheresis procedures to collect sufficient cells for transplant. Consequently, at the University of Nebraska Medical Center, a rule was introduced that excluded blood stem cell harvesting and concurrent radiation therapy.

With the advent of mobilized blood stem cell harvesting, this issue was revisited. If radiation did not affect stem cell collection quality, appropriate patients could undergo debulking radiation therapy while having blood stem cells collected, providing time efficiency in therapy. Unexpectedly, partial-body irradiation concurrent with attempts to mobilize blood stem cells inhibited mobilization. This does not appear to be a nonspecific effect of partial-body irradiation, as it occurred following irradiation of three different areas. Because all the mice survived these irradiations, no selection bias was introduced. The inhibition of blood stem cell mobilization appeared to be mediated by a circulating factor present in the plasma of lower hemibody irradiated mice. The plasma of upper hemibody or limb only irradiated mice has not yet been assayed for this inhibitory factor. Additionally, several puzzling disassociations of behavior of mature hematopoietic cells and progenitor cells were noted. The WBC count was increased by the cytokine injection in all except the lower hemibody irradiated mice, even though progenitor cell numbers were not increased. Inclusion of the spleen in the irradiation field eliminated the increase in WBC count.

Based on these observations and an anecdotal report of abscopal depression of G-CSF-mobilized blood stem cells in a patient following local radiation therapy for lymphoma (5), a rule limiting administration of radiation therapy near the time of collection of cytokine-mobilized blood stem cell harvests appears prudent. Obviously, the characteristics of the factor responsible for inhibition of blood stem cell mobilization are of considerable interest, as it may be possible to inactivate this factor, thus allowing patients to more readily undergo blood stem cell collection.

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Appendix 9b  
**Mobilization of Blood Stem Cells**

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**Key Words.** *Blood stem cells · Transplantation · Mobilization inhibition · Hematopoietic recovery*

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### **ABSTRACT**

Deliberately increasing the number of hematopoietic stem cell and progenitors in the circulation allows faster and more efficient collection of sufficient cells for transplantation in both the allogeneic and autologous settings. These mobilized stem cells, when transplanted, provide quicker hematopoietic recovery for the patient than do nonmobilized blood stem cells or steady-state marrow-derived stem cells. Currently used clinical procedures to produce stem cell mobilization include administration of G-CSF or GM-CSF, either as single agents or in combination with myelosuppressive chemotherapy. Some autologous blood stem cell donors exhibit indifference to currently applied mobilization therapies. This failure to mobilize has been associated with

prior stem cell toxic therapy, e.g., radiation therapy and chemotherapy, but the association is incomplete. The observation that occasional normal donors have failed to respond to mobilization therapy indicates that factors other than stem cell damage could also be involved. Recently, a murine model has provided evidence that a circulating factor inhibits mobilization in some settings. Preliminary investigations have suggested that a circulating factor may inhibit mobilization of human hematopoietic progenitor cells in some instances. Studies to identify this factor(s) are underway. The mechanisms of blood stem cell mobilization are still poorly understood and there continues to be the potential to improve this process. *Stem Cells* 1997;15(suppl 3):##-##

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### **INTRODUCTION**

Manipulations designed to increase the number of human hematopoietic stem and progenitor cells in the circulation of potential peripheral blood stem/progenitor cell donors has not only made collection of these cells with apheresis a more efficient process, but also has produced a graft product which restores hematopoiesis more rapidly than steady-state marrow cell grafts [1]. Mobilization techniques currently considered standard for clinical use include administration of myelosuppressive chemotherapy and a growth factor which affects the granulocyte lineage (sargramostim [2] or filgrastim [3]) or administration of either of these growth factors alone [1, 4]. Occasionally, autologous donors fail to respond to mobilization-inducing therapies. Factors which sometimes predict for failure to mobilize have included prior chemotherapy and radiation therapy [5]. Although some patients previously treated with chemotherapy and/or radiation therapy exhibit vigorous mobilization in response to growth factors and/or myelosuppressive chemotherapy, these associations have led to the understandable assumption that failure to mobilize is in some way related to a damaged stem cell pool. However, the recent advent of allogeneic peripheral blood stem cell transplantation has revealed that an occasional normal donor fails to mobilize following growth factor administration [6], suggesting that factors other than, or in addition to, stem cell damage may be involved when indifference to mobilization occurs. Because a murine

mobilization study suggested that a circulating factor could play a role in the etiology of failure to respond to mobilizing therapy [7], a study was undertaken to determine if a circulating factor(s) might influence the magnitude of response to clinical mobilization attempts.

## MATERIALS AND METHODS

### Murine Assay Model

A murine model of mobilization inhibition induced by partial body irradiation was developed [7], whereby injection of mobilizing growth factors failed to result in a large increase of circulating progenitors if 2 Gy of partial body irradiation (lower limbs, upper hemibody or lower hemibody) was administered on the same days as the growth factors. Table 1 shows the significant degree of mobilization of colony-forming cells (CFU) in the blood and spleen and the slight increase in femoral marrow CFU in recipients of growth factors only. Upper hemibody radiation (XRT) greatly inhibited mobilization of CFU into the blood and spleen as did lower limb XRT. Both lower limb and lower hemibody XRT completely inhibited mobilization of CFU into the blood.

Since the effect occurred regardless of the site irradiated, the inhibition was suspected to be systemic. To test this premise, plasma from hemibody irradiated mice was injected i.v. into untreated mice 10 min before receiving the mobilizing growth factors. The effect on mobilization of CFU to the spleen 24 h later was assessed compared to recipients of either saline or normal donor plasma. Plasma from part-body irradiated mice inhibited blood stem cell mobilization [7] to a level of 16% of that observed in the control. The hypothesis was formulated that radiation therapy released or activated a circulating inhibitor of blood stem cell mobilization. This murine inhibition model was then adapted for the current study to demonstrate proof of principal that a circulating mobilization-inhibiting factor could be functioning in autologous and normal donors who failed to mobilize well.

### Human Subjects

Five individuals, three autologous donors undergoing mobilizing growth factor administration for blood stem cell collection with poor mobilization responses and two normal volunteers, were identified. Following administration of filgrastim 10 µg/kg s.c. for mobilization for five days, and for one patient erythropoietin (EPO) 300 U/kg for five days along with the filgrastim, these patients required 9, 10 and 4 apheresis procedures to collect 0.59, 1.12, and  $0.37 \times 10^6$  CD34<sup>+</sup> cells/kg, respectively. After informed consent was given by the participants, heparinized plasma was obtained from a blood sample of each person. This study was approved by the University of Nebraska institutional review board.

**Table 1. Impact of lower limb (LL), upper hemibody (UHB) or lower hemibody (LHB) irradiation (XRT) on growth factor-induced mobilization in murine blood, femoral marrow, and spleen.**

Peak mobilization (Fold increases over nonmobilized) for each treatment <sup>a</sup>				
	Growth factor only <sup>b</sup>	Growth factor plus LL-XRT	UHB-XRT	LHB-XRT
Blood	×20	none	×4	none
Spleen	×36	×10	×5	irradiated <sup>c</sup>
Femoral marrow	×3	irradiated <sup>c</sup>	×3	irradiated <sup>c</sup>

<sup>a</sup> Colony-forming cells (CFU-GM, CFU-mix, HPP-CFC) were assayed before and after treatment. The average peak fold increase is presented. Growth factor was administered daily each morning, and a 2 Gy fraction of XRT each afternoon for five days. Peak mobilization occurred after three to five days of treatment.

<sup>b</sup> 500 U/kg EPO plus 15 µg/kg filgrastim.

<sup>c</sup> This tissue was included in this irradiation field; therefore, colony-forming cell numbers were decreased from the control value.

**Mice**

The study employed young adult female Balb/c mice purchased from Charles River. The mice were maintained on a 12-h light, 6:00 a.m. to 6:00 p.m., 12-h dark cycle and provided both sterilized food and acidified (pH2) sterile water ad libitum. They were maintained in filter-top cages in laminar airflow cabinets.

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**Growth Factors**

Recombinant EPO 500 U/kg (Ortho Biotech) and filgrastim 15  $\mu$ g/kg (Amgen) were used as the mobilizing regimen for the murine mouse model.

**Hematopoietic Colony Assay**

Mobilization of stem cells was assessed by in vitro hematopoietic colony-forming assay; high proliferative potential-colony-forming cells (HPP-CFC). Briefly, at necropsy, spleens were removed aseptically and cells were gently teased from the spleens with 25 gauge needles into Hank's balanced salt solution without calcium and magnesium. Clumps were dispersed by repeated aspiration with a 1 cc syringe without a needle. Cells were washed once and resuspended in Tris-buffered ammonium chloride (ACT) for 5 min to lyse mature red blood cells. After 5 min, an equal volume of complete medium containing serum was added to halt the action of the ACT. The cells were washed once and resuspended in Hank's balanced salt solution without calcium and magnesium. These cells were then enumerated and employed in the colony assay detailed below.

**HPP-CFC Assay**

Spleen ( $1 \times 10^5$ ) cells were plated in 60 mm dishes containing Iscove's modified Dulbecco's medium, 0.3% agar, 15% fetal bovine serum, 100 units penicillin, 100  $\mu$ g streptomycin, 50  $\mu$ M 2-mercaptoethanol and 10ng interleukin 3 (IL-3) with 10% L cell conditioned medium (or recombinant M-CSF) as sources of colony-stimulating activity. Dishes were incubated for 11 days at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> in air. The colonies were enumerated using an inverted microscope. Microscopic colonies containing 50 cells and less and greater than 2 mm diameter were counted.

**Data Analysis**

Means and standard deviations were calculated when possible. One tailed Student's *t* test was employed to determine significant differences.

**Experimental Design**

Plasma, 0.2 ml, from either autologous donors receiving filgrastim for mobilization and identified as poor mobilizers or from normal volunteers or 0.2 ml plasma harvested from mice after receiving hemibody irradiation as described above was injected into nonirradiated control Balb/c mice 10 min prior to injection of EPO, 500 U/kg and filgrastim, 15  $\mu$ g/kg. The mobilization response was determined in the murine assay system by measuring HPP-CFC in the spleen of the animals using methods described above.

**RESULTS**

The effect of the plasma collected from the various sources listed above on mobilization in the mouse treated with EPO and filgrastim is listed in Table 2. Using the one-tailed Student's *t* test, the difference between the mobilization observed in the mice treated with plasma from poorly mobilizing patients and the growth factor-treated control mice was statistically significantly different ( $p \leq .005$ ). The mice treated with plasma from normal volunteers also had significantly different mobilization than the mice injected with plasma from the poorly mobilizing patients ( $p \leq .025$ ). The difference in mobilization between the control mice and the mice pretreated with plasma from previously irradiated mice was significant ( $p \leq .01$ ), but mobilization in the mice treated with plasma from normal volunteers was not significantly different from that observed in the control mice.

**Table 2. Impact of plasma from normal volunteers, partial-body irradiated mice and poorly mobilizing cancer patients on growth factor mobilization in the mouse**

Plasma source <sup>a</sup>	Mobilization (% of saline control) <sup>b</sup>
None, growth factor + saline only	100 ± 14
Plasma from partial-body irradiated mice <sup>c</sup>	29 ± 13
Plasma from normal volunteers	72 ± 13
Plasma from poorly mobilizing patients	22 ± 12

<sup>a</sup>0.2 ml heparinized plasma injected i.v. Plasma was injected at mid-morning 10 min prior to growth factor (500 µ/kg EPO plus 15 µg/kg G-CSF) injection.

<sup>b</sup>Mobilization was assessed as HPP-CFC per 1 × 10<sup>5</sup> spleen cells, 24 h following growth factor only or growth factor and plasma injection. Mean values ± standard deviations are presented.

<sup>c</sup>Partial body irradiation of mice inhibits blood stem cell mobilization via a circulating inhibitor. This was employed as a control for positive inhibition.

## DISCUSSION

Some patients undergoing growth factor-mobilized autologous blood stem harvests do not mobilize well and require more than the minimum anticipated number of leukaphereses to collect a suitable graft product. Patients with prior exposure to chemotherapy and/or radiation therapy were more likely to be poor mobilizers, and damaged stem cells were believed to play a role in poor mobilization. However, some normal donors with no history of stem cell toxic prior therapy have also exhibited poor mobilization. Given that a circulating inhibitor of blood stem mobilization had been detected in partial-body irradiated mice, the mouse assay of inhibition of blood stem cell mobilization was adapted to assay plasma from normal donors, poorly mobilizing normal donors and cancer patients. The preliminary results of these assays (Table 2) suggested that plasma from normal donors caused some inhibition (to 72% of the control). Inhibition resulting from injection of plasma collected from normal poorly mobilizing donors was noted, as mobilization in the animal model was 76% of the control value. The plasma from the poorly mobilizing cancer patients was even more inhibitory (to 22% of the control) at a level comparable to that of plasma from partial-body irradiated mice. These results lead to the suggestion that an inhibitor of blood stem cell mobilization may be found in the plasma of some cancer patients and normal donors. Whether this is the same inhibitor or similar to that found in the circulation of mice following radiation therapy remains to be defined.

A recent study of different wild-type murine strains [8] revealed that genetic influences may play a role in the response to growth factor-induced mobilization attempts. The patterns of mobilization observed included rapid vigorous response, intermediate response and indifference to growth factor administration (e.g., inhibition) depending upon the genetic strain being examined.

The nature of the inhibition is obviously of considerable interest, as is the mechanism of action. Recently, inhibitors of growth factor signaling have been described [9-11]. Some of these inhibitors are growth factor-induced, suggesting that a negative feedback loop may be operational. Potentially, endogenous cytokines induced by irradiation of mice, induced by cytotoxic chemotherapy, radiation therapy or the presence of malignancy in cancer patients and induced by virtue of genetic constitution in response to an underlying relatively benign event, such as a recent infection in some normal donors, may be responsible for this inhibition of blood stem cell mobilization. Obviously, the ability to detect patient donors and/or normal donors with the active circulating inhibitor and the ability to neutralize this inhibitor would increase the efficiency and cost-effectiveness of blood stem cell collection for these donors.

Currently, new combinations of growth factors which potentially tailor the cellular content of the harvest for therapeutic purposes, e.g., IL-2-responsive cells, dendritic cells, are being pursued. These developments, as well as a better understanding of the mechanisms of blood stem cell mobilization, offer the potential for further improvements in the application of this procedure.

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